

# Phevamine A, a small molecule that suppresses plant immune responses

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**Bacterial plant pathogens cause significant crop damage worldwide. They invade plant cells by producing a variety of virulence factors, including small-molecule toxins and phytohormone mimics. Virulence of the model pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*) is regulated in part by the sigma factor HrpL. Our study of the HrpL regulon identified an uncharacterized, three-gene operon in *Pto* that is controlled by HrpL and related to the *Erwinia* *hrp*-associated systemic virulence (*hsv*) operon. Here, we demonstrate that the *hsv* operon contributes to the virulence of *Pto* on *Arabidopsis thaliana* and suppresses bacteria-induced immune responses. We show that the *hsv*-encoded enzymes in *Pto* synthesize a small molecule, phevamine A. This molecule consists of L-phenylalanine, L-valine, and a modified spermidine, and is different from known small molecules produced by phytopathogens. We show that phevamine A suppresses a potentiation effect of spermidine and L-arginine on the reactive oxygen species burst generated upon recognition of bacterial flagellin. The *hsv* operon is found in the genomes of divergent bacterial genera, including ~37% of *P. syringae* genomes, suggesting that phevamine A is a widely distributed virulence factor in phytopathogens. Our work identifies a small-molecule virulence factor and reveals a mechanism by which bacterial pathogens overcome plant defense. This work highlights the power of omics approaches in identifying important small molecules in bacteria–host interactions.**

natural products | genome mining | phytopathogen | virulence factor | plant immunity

**B**acterial small molecules play key roles in pathogen–plant interactions. *Pseudomonas syringae*, a phylogenetically diverse species of bacteria responsible for many crop diseases, employs a myriad of virulence factors, including secreted protein effectors delivered to host cells by the type III secretion system (T3SS) and small molecules (1, 2). Coronatine, syringomycin, syringopeptin, tabtoxin, and phaseolotoxin are among the small-molecule virulence factors produced by various strains of *P. syringae*, acting as phytohormone mimics or phytotoxins (1). For example, coronatine disrupts plant immune signaling by mimicking the structure of the plant hormone jasmonic acid-isoleucine (1, 3), and phaseolotoxin contributes to virulence by interfering with the synthesis of L-arginine and polyamines in plants (4, 5). While many protein effectors have been extensively studied in *P. syringae*, only a limited number of small molecules have been identified. The genomes of *P. syringae* and other phytopathogens encode potential biosynthetic enzymes (6, 7). Identifying the cryptic small molecules synthesized by these enzymes may reveal new virulence mechanisms that could be targeted to control plant infection.

Many phytopathogenic virulence factors function to suppress plant immune responses (8, 9). The first layer of the plant immune system relies on the recognition of conserved microbe-associated molecular patterns (MAMPs) by host pattern recognition receptors to generate MAMP-triggered immunity (MTI) (10). The MTI signaling cascade includes receptor kinase activation, reactive oxygen species (ROS) production, calcium influx, mitogen-activated protein kinase activation, defense gene activation, and callose deposition at

the plant cell wall (*SI Appendix, Fig. S1*); each of these steps can be targeted by virulence factors (10). The sigma factor HrpL controls the expression of *P. syringae* virulence factors including the T3SS and its associated effector genes (11). HrpL also regulates genes responsible for the biosynthesis of coronatine in some, but not all, *P. syringae* strains (12, 13). Our recent study identified a number of uncharacterized HrpL-regulated genes that encode putative biosynthetic enzymes for small molecules (12). We therefore proposed that these HrpL-regulated genes are responsible for synthesizing small molecules that play a role in virulence (12).

In this study, we focus on an HrpL-regulated three-gene biosynthetic operon *PSPTO\_0873–0875* from *P. syringae* pv. *tomato* DC3000 (*Pto*) (12, 14). This operon shares 66–85% homology with *hrp* (hypersensitive response and pathogenicity)-associated systemic virulence (*hsv*) genes from *Erwinia amylovora* (15). The *hsv* operon was implicated in the virulence of *E. amylovora* on apple shoots (16), but the small molecule(s) produced by *hsv*-encoded enzymes are unknown. Here, we confirm that the *Pto* *hsv* operon acts as a *P. syringae* virulence factor on *Arabidopsis thaliana* and demonstrate that the *hsv* operon dampens host immune responses in both *A. thaliana* and *Nicotiana benthamiana*. We demonstrate that the enzymes encoded by the *hsv*

## Significance

**Bacterial pathogens cause plant diseases that threaten the global food supply. To control diseases, it is important to understand how pathogenic bacteria evade plant defense and promote infection. We identify from the phytopathogen *Pseudomonas syringae* a small-molecule virulence factor—phevamine A. Both the chemical structure and mode of action of phevamine A are different from known bacterial phytotoxins. Phevamine A promotes bacterial growth by suppressing plant immune responses, including both early (the generation of reactive oxygen species) and late (the deposition of cell wall reinforcing callose in leaves and leaf cell death) markers. This work uncovers a widely distributed, small-molecule virulence factor and shows the power of a multidisciplinary approach to identify small molecules important for plant infection.**

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The authors declare no conflict of interest.

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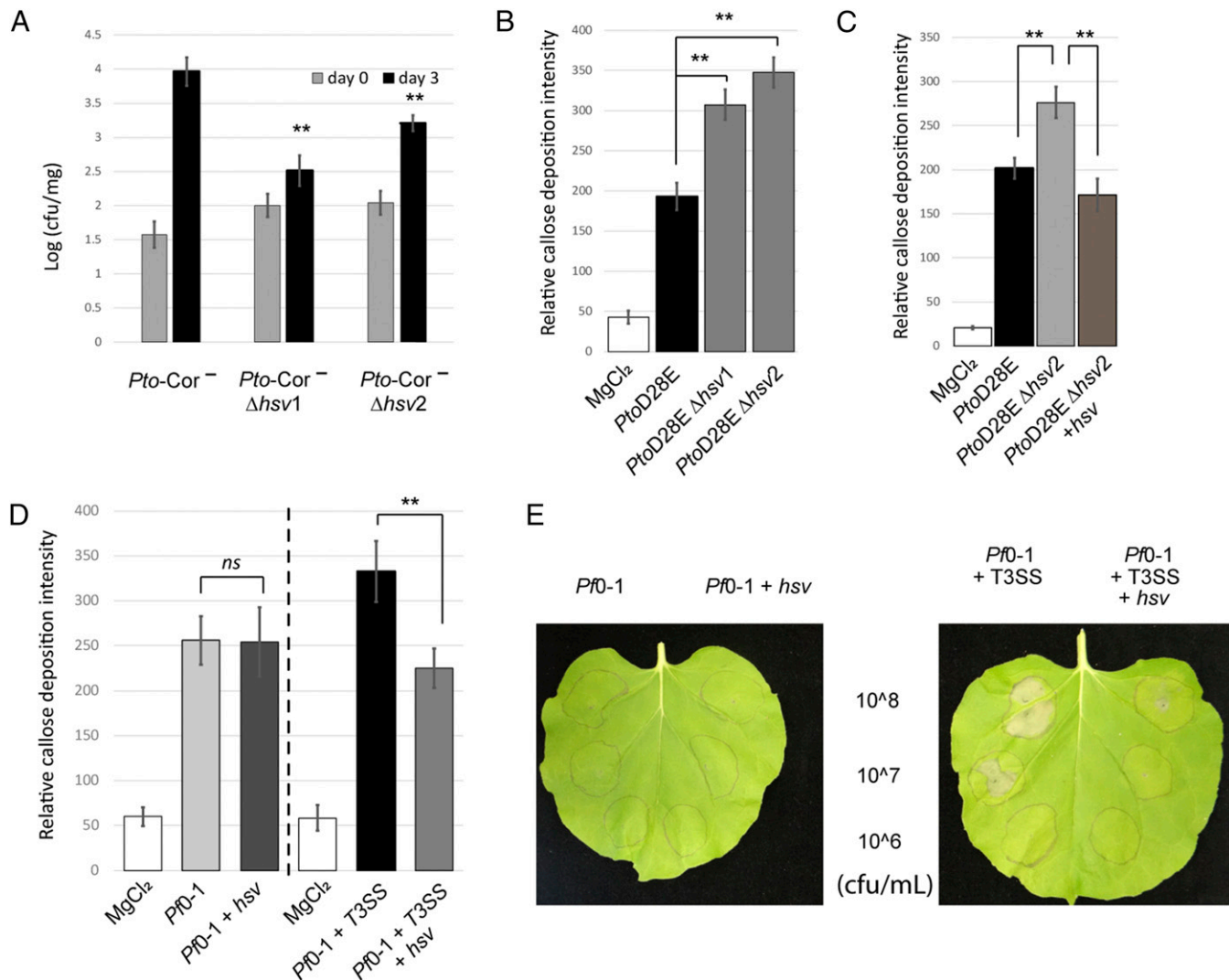
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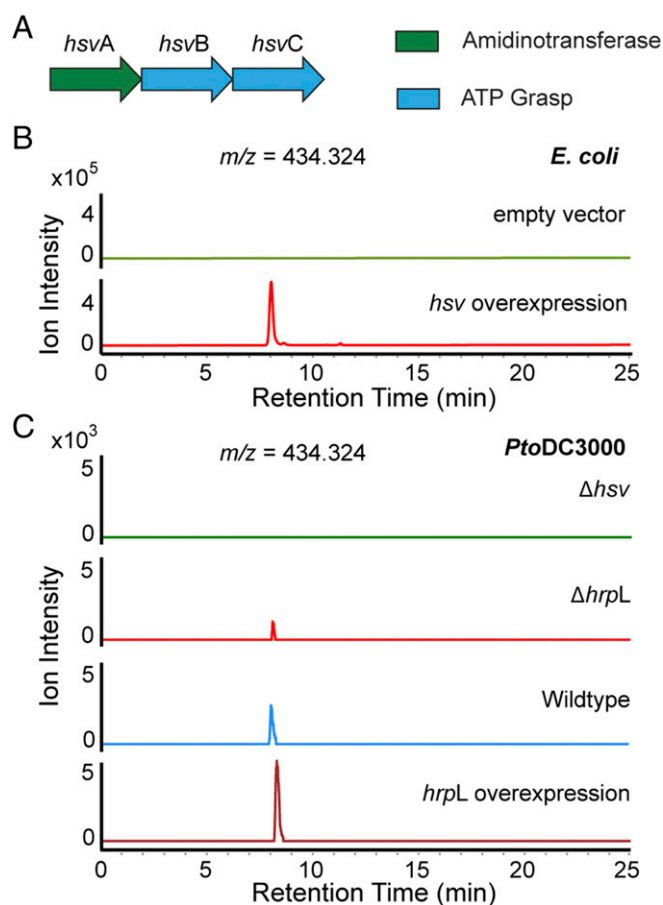
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**Fig. 2.** Metabolomics reveals the small molecules synthesized by *hsv*-encoded enzymes. (A) Graphical representation of the *hsv* operon. (B) Comparative metabolomics analysis in *E. coli*. Overexpression of *hsv* produces a molecule with an  $m/z$  of 434.324 (green), which is absent in *E. coli* harboring the empty vector (red). (C) Comparative metabolomics analysis in *Pto*. The molecule with the  $m/z$  of 434.324 is absent in  $\Delta hsv$  (green) and reduced in  $\Delta hrpL$  (red), but present in both wild-type *Pto* (blue) and *Pto* overexpressing *hrpL* (maroon). Extracted ion chromatograms for 434.324 are shown in B and C. The slight change in retention time is due to the hydrophilicity of this molecule. Experiments presented in B and C were repeated at least three times.

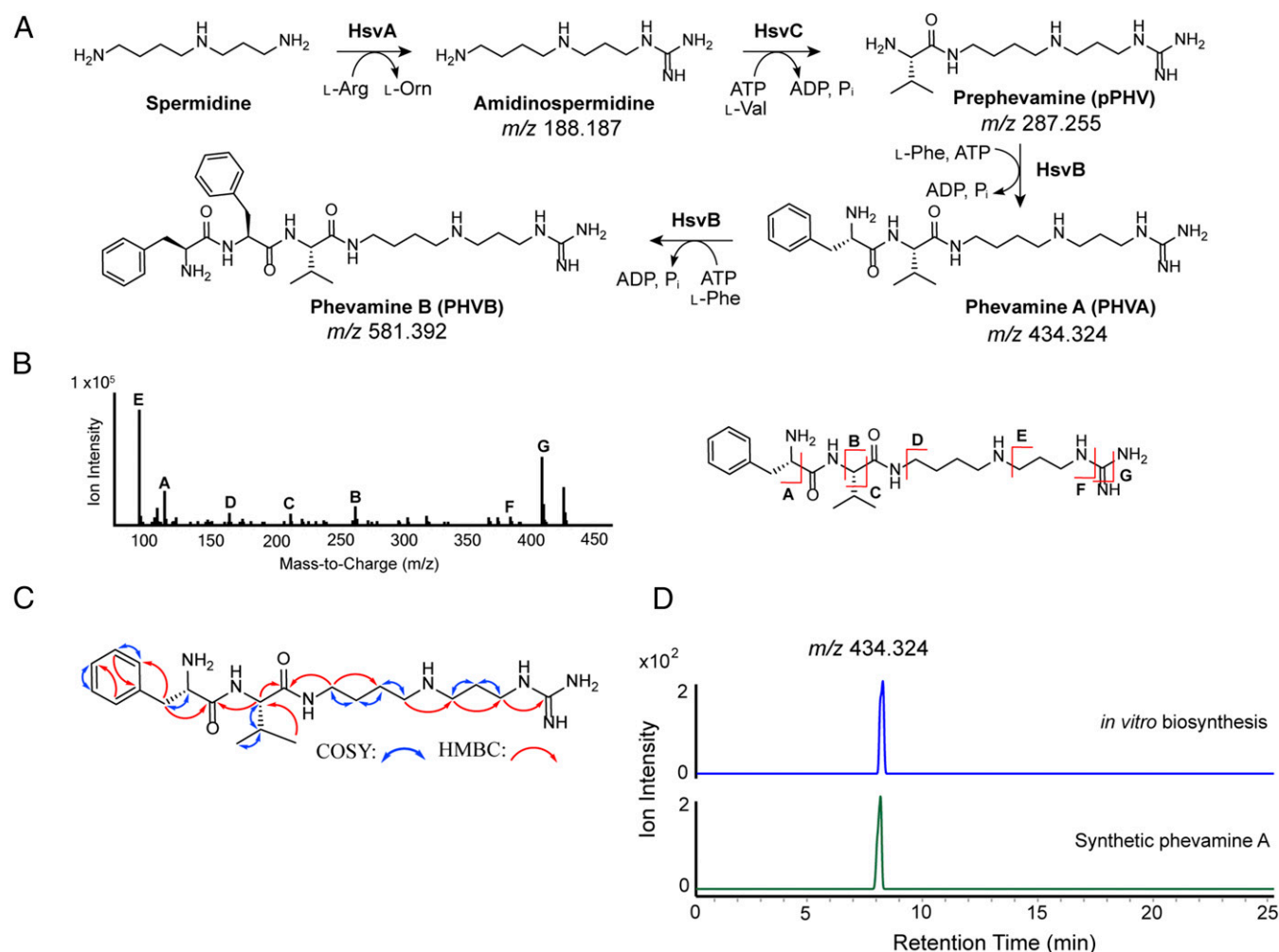
and in vitro isolated phevamine A exhibit identical  $^1H$  and  $^{13}C$  NMR signals, and the same LC retention time and MS fragmentation pattern as the species detected in the bacterial culture extract (Fig. 3D and *SI Appendix*, Figs. S14–S22). These results confirmed the structure assignment for phevamine A and provided materials for biological activity testing of this molecule. In an initial activity test, we examined the ability of phevamine A to bind ferric iron using a chromeazuroil S assay and observed no significant binding to ferric iron (*SI Appendix*, Fig. S23), suggesting phevamine A is unlikely acting as a siderophore.

**Comparative Genomics Suggests a Potential Host Target for Phevamines.** We investigated the phylogenetic distribution of *hsv* in bacterial genomes using MultiGeneBlast that identifies homologous gene clusters based on sequence similarity and gene synteny (34). We found that *hsv* is widely distributed across plant-pathogenic bacterial genera including *Pseudomonas*, *Erwinia*, and *Pantoea*. We compared the distribution of the *hsv* operon to those biosynthetic gene clusters encoding known *P. syringae* phytotoxins, focusing on coronatine, mangotoxin, syringolin, phaseolotoxin, and tabtoxin (Fig. 4A and *Dataset S1*) (1, 35). The *hsv* operon is present in

~37% of *P. syringae* (107 out of 292) and is the most widely distributed gene cluster of the six analyzed. The *hsv* operon rarely cooccurs with any of the aforementioned small-molecule biosynthetic clusters. This anticorrelation is especially prominent between *hsv* and phaseolotoxin biosynthetic genes in the genomes of otherwise extremely closely related strains of *P. syringae* pv. *actinidiae* (36). We previously showed that functionally redundant virulence factors rarely cooccur in the same strain (37). Specifically, the small molecule coronatine and three different type III secretion system protein effectors use four distinct mechanisms to target the same host defense signaling pathway, and only genes responsible for a single mechanism are typically found in any given *P. syringae* genome (37). Based on the clear anticorrelation of *hsv* with the phaseolotoxin biosynthetic genes, and the knowledge that phaseolotoxin targets host arginine and polyamine biosynthesis by inhibiting ornithine carbamoyltransferase and ornithine decarboxylase (Fig. 4B) (4, 5, 38), we hypothesized that the action of phevamine A involves polyamines and/or arginine. This hypothesis is consistent with previous implication of polyamine function in plant immune responses (39, 40). Furthermore, our genomic analysis revealed that many *P. syringae* strains do not contain the biosynthetic gene clusters for phevamine A or the five other phytotoxins described herein (Fig. 4A). These strains could produce different small molecules that might play a role in bacterial-plant interaction.

**Polyamines and Arginine Potentiate an Early MTI Response.** We investigated the effect of arginine and polyamines on the MTI response induced by the well-studied MAMP flg22, a short peptide derived from the *Pseudomonas* flagellin (18). We monitored the ROS burst following recognition of flg22 by the pattern recognition receptor FLS2 (*SI Appendix*, Fig. S1) (41, 42). The polyamines putrescine, spermidine, and spermine potentiated the flg22-induced ROS burst in *N. benthamiana*, resulting in an earlier and larger amplitude response (Fig. 5A and B and *SI Appendix*, Fig. S24A). The potentiation was dose-dependent (*SI Appendix*, Fig. S24B). Spermidine alone induced only a slight ROS burst (*SI Appendix*, Fig. S24C) (40), and the increase of the flg22-induced ROS burst in the presence of spermidine was higher than an additive effect (*SI Appendix*, Fig. S24C). The potentiation by spermidine was also observed in *A. thaliana*, but was not observed in the flg22 receptor mutant *fls2* (Fig. 5C and *SI Appendix*, Fig. S25A and B). Spermidine potentiation was also observed using the elicitor elf18 (*SI Appendix*, Fig. S25C) (43). Surprisingly, the *Arabidopsis*  $Ca^{2+}$  burst induced by flg22 was reduced in the presence of spermidine (Fig. 5D and *SI Appendix*, Figs. S1 and S25D). Arginine had a similar effect on ROS burst in *A. thaliana* and *N. benthamiana*, while L-citrulline did not (*SI Appendix*, Figs. S26 and S27). Thus, spermidine and arginine potentiate MAMP-induced ROS bursts in both plant species, and spermidine modulates the flg22-induced  $Ca^{2+}$  burst, at least in *A. thaliana*. These results are consistent with previously proposed functions of polyamines in plant defense responses (39, 40), including up-regulation of polyamine biosynthesis in plants following challenge with bacterial pathogens (44–47), subsequent polyamine transport to the apoplast (44), and increased resistance toward *Pseudomonas viridiflava* observed after exogenous addition of spermine (47).

**Phevamine A Suppresses Spermidine and Arginine Potentiation of the flg22-Induced ROS Burst.** The anticorrelation of phevamine A- and phaseolotoxin-encoding genes suggested that phevamines might impair the effect of spermidine and arginine on the early MAMP-dependent ROS burst. We therefore tested whether phevamine A could impair the effect of exogenously supplied spermidine and arginine in the MTI-induced ROS burst potentiation assay. Leaf disks of *N. benthamiana* were challenged with flg22 in the presence of phevamine A, spermidine, or both (Fig. 5A). We observed



**Fig. 3.** Structural characterization and biosynthesis of prepheavamine and the phevamines. (A) Biosynthetic pathway of prepheavamine and the phevamines based on in vitro characterization. (B) Structural analysis of phevamine A produced by *E. coli* overexpressing *hsv* using tandem MS. (C) COSY and  $(^1H,^{13}C)$ -HMBC correlations of in vitro synthesized and purified phevamine A. (D) Extracted ion chromatograms of phevamine A ( $m/z$ , 434.324) produced via in vitro enzymatic synthesis (blue) or total chemical synthesis (green).

that phevamine A suppressed the spermidine potentiation of the flg22-induced ROS burst in *N. benthamiana*, but did not directly affect the flg22 response (Fig. 5A). We noted that prepheavamine also suppressed the spermidine potentiation of the flg22-induced ROS burst, but phevamine B did not (Fig. 5B). This result is consistent with our observation that phevamine B is not naturally produced by *Pto* (SI Appendix, Fig. S6). In contrast, only phevamine A suppressed the spermidine-mediated flg22-induced ROS burst in *A. thaliana* leaf disks in a similar experimental setup (Fig. 5C). This indicates that the molecular mechanism(s) of the spermidine potentiation might differ slightly between *N. benthamiana* and *A. thaliana*.

Spermidine affects both the flg22-induced ROS and  $Ca^{2+}$  bursts (Fig. 5D and SI Appendix, Fig. S24C). We therefore tested the effect of phevamine A on the flg22-induced  $Ca^{2+}$  burst in the presence and absence of spermidine using a transgenic *A. thaliana* line that expresses the aequorin reporter (48). As noted above, spermidine suppressed the flg22-induced  $Ca^{2+}$  burst, but the addition of phevamine A did not affect this suppression. Phevamine A also had no direct effect on the  $Ca^{2+}$  burst induced by flg22 (Fig. 5D). These data suggest that phevamine A acts downstream of the  $Ca^{2+}$  burst.

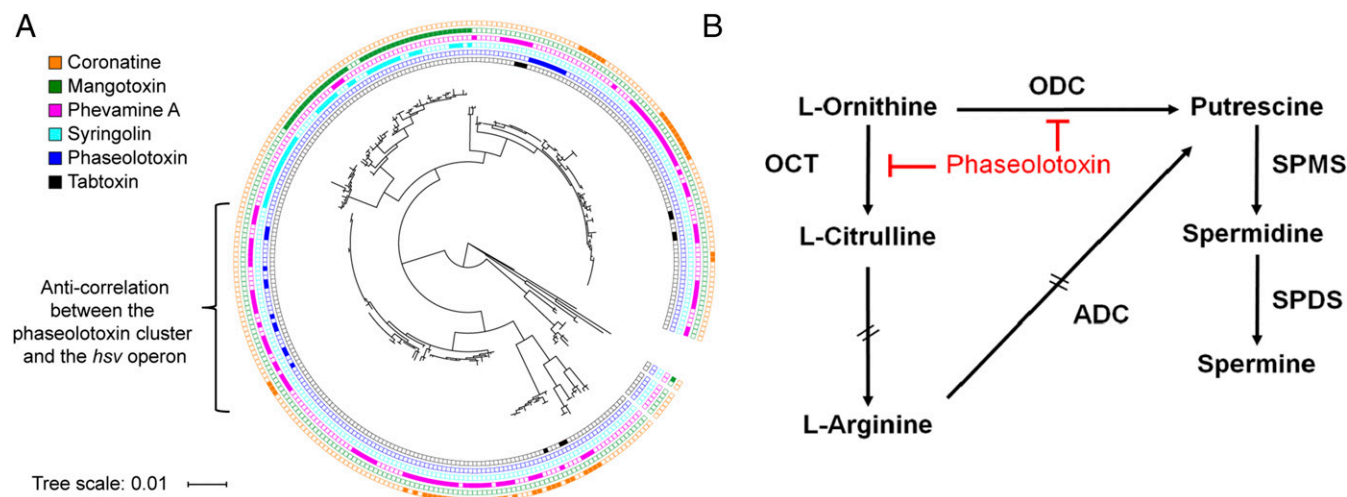
Additionally, both phevamine A and prepheavamine were tested for their effect on the ROS potentiation mediated by arginine in

both *N. benthamiana* and *A. thaliana*. Phevamine A, but not prepheavamine, could suppress the arginine-mediated potentiation of the flg22-induced ROS burst in *N. benthamiana* (SI Appendix, Fig. S27). This may imply that the potentiation mediated by spermidine and arginine in *N. benthamiana* involves slightly divergent molecular mechanism(s), supported by the different potentiation phenotypes for prepheavamine. Similarly, only phevamine A and not prepheavamine could inhibit the arginine potentiation of the flg22-induced ROS burst in *A. thaliana*.

Overall, we observed that phevamine A consistently suppressed both the spermidine- and arginine-mediated potentiation of the flg22-induced ROS burst across two divergent plant species. Thus, phevamine A is likely the most physiologically relevant small molecule produced by the *hsv* operon in *Pto*.

## Conclusions

We identify a bacterial small-molecule virulence factor, phevamine A. The biosynthesis of phevamine A is controlled by the HrpL virulence regulator in *P. syringae*. Interestingly, phevamine A shares structural similarity with insect polyamine toxins: argiotoxin from the orb-weaver spider, and philanthotoxin from the Egyptian solitary wasp, both of which contain a polyamine and two amino acids, and are powerful neurotoxins that target



**Fig. 4.** Distribution of the *hsv* operon and other toxins among *P. syringae* and graphical representation of polyamine biosynthesis and action of phaseolotoxin. (A) Phylogenetic tree of 292 *P. syringae* strains and the distribution of coronatine, mangotoxin, phevamine A, syringolin, phaseolotoxin, and tabtoxin biosynthetic genes, from the outer ring to the inner ring. The tree scale represents the nucleotide substitution per site. (B) Phaseolotoxin inhibits the arginine and polyamine pathways by suppressing the activity of ornithine decarboxylase (ODC) and ornithine carbamoyltransferase (OCT). ADC, arginine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase. Double dashed arrows represent pathways with intermediates not shown. Reproduced from ref. 60 with permission from American Society for Microbiology.

ion channels (49, 50). The structural similarity suggests related mechanisms, which will be the focus of future studies.

The importance of phevamine A in bacterial virulence has been demonstrated in two different pathogens, *E. amylovora* (16) and *P. syringae* in this study. We show that the *hsv* operon and purified phevamine A can suppress both early and late markers of plant immune responses. Specifically, the *hsv* operon suppresses T3SS/harpin-induced callose deposition and host cell death. The phylogenetic distribution of the *hsv* operon suggested that phevamine A is a conserved virulence factor among bacterial phytopathogens. The anticorrelation of *hsv* and the biosynthetic genes for phaseolotoxin within a closely related clade of *P. syringae* pv. *actinidiae* genomes suggested functional redundancy between phevamine A and phaseolotoxin. We demonstrated that phevamine A impairs plant immune signal potentiation by polyamines and arginine, while it is known that phaseolotoxin targets the synthesis of polyamines and arginine.

Our discovery of phevamine A also provided the opportunity to describe functions for polyamines in the potentiation of the MAMP-induced ROS burst induced by flg22, expanding the role of polyamines as important defense mediators. Our results are consistent with a model (*SI Appendix*, Fig. S28) in which *P. syringae* uses either phaseolotoxin or phevamine A to dampen host immune output by altering polyamine biosynthesis or the signaling capacity of polyamines, respectively. We speculate that components of the T3SS and/or harpins may be recognized at the cell surface, acting as elicitors of MTI subject to polyamine potentiation and suppression by phevamine A.

We show that two ATP-grasp enzymes are responsible for assembling phevamine A. These enzymes belong to a large superfamily of enzymes that catalyze amide bond formation by activating carboxylic acids through phosphorylation (51). ATP-grasp-type enzymes are widely used by bacteria for the synthesis of diverse small molecules, including the virulence factors phaseolotoxin, dapdiamide, and mangotoxin (52–54). Studies of these enzymes in a marine proteobacterium and a soil actinomycete have led to the discovery of small molecules with distinct structures but unknown functions (55, 56). Still, most biosynthetic operons containing ATP-grasp-type enzymes have eluded genome-guided discovery. Our study suggests that targeted mining of

uncharacterized ATP-grasp enzymes will likely lead to novel small molecules.

Our approach for identifying cryptic small molecules builds on the rich knowledge of pathogen gene expression and integrates comparative genomic analysis, biochemical enzyme reconstitution, and physiological assays. Bacterial genomes harbor many biosynthetic operons of unknown function; therefore, this approach holds potential for identifying many more small molecules essential for bacteria–host interactions.

## Methods

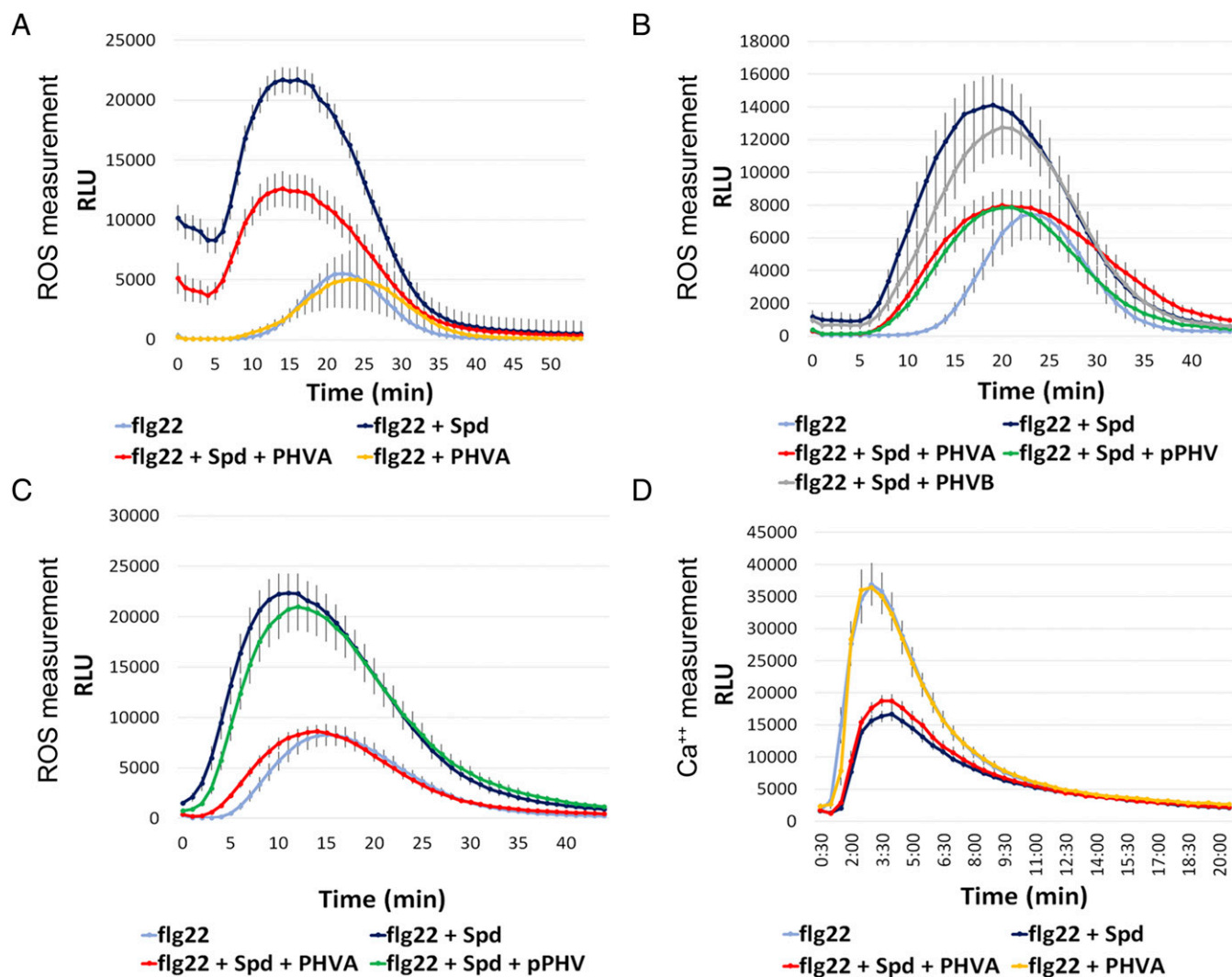
**Metabolite Extraction for Metabolomics.** *E. coli*, *P. syringae*, and *P. fluorescens* cultures (100 mL) were spun down at 3,500 × *g* and 4 °C for 10 min. Solvent extractions were performed using an equal volume of supernatant to chloroform-methanol (2:1:1). The top aqueous layer was separated into a round-bottom flask and concentrated under vacuum. The dried-down material was resuspended and transferred to a small glass vial, concentrated under reduced pressure, and stored at –20 °C until MS analysis.

**MS and MS/MS Analysis.** Culture extracts, in vitro synthesized phevamines, and chemically synthesized phevamine A, were analyzed by Agilent 6520 accurate-mass quadrupole-time of flight (Q-TOF) LC/MS using a Phenomenex Kinetex 5-μm C18 column (100 Å, 150 mm × 4.60 mm). Mobile phases were water and acetonitrile, each containing 0.1% formic acid. The gradient was held at 2% acetonitrile for 2 min before ramping up to 45% acetonitrile over 17 min at a flow rate of 0.4 mL/min. For each sample, *m/z* 434.324 was selected at a retention time of 8 ± 5 min and analyzed by MS fragmentation. Targeted MS/MS was conducted using 30-V collision energy, 970.9-ms/s acquisition time, and a 4 *m/z* isolation width.

**Preparative Scale in Vitro Enzymatic Synthesis of Phevamines.** One milliliter, one-pot enzymatic assays for phevamine A were conducted at room temperature for 2 h. Assays contained 5 mM L-arginine, 5 mM spermidine, 1 mM L-valine, 0.5 mM L-phenylalanine, 100 mM Hepes (pH 7.5), 2 mM MgCl<sub>2</sub>, 1 mM ATP, 20 μM HsvA, 10 μM HsvB, 10 μM HsvC, and water. The reaction was then quenched with 1 mL acetonitrile to precipitate protein overnight at –20 °C. The precipitated proteins were removed by centrifugation. Phevamines were purified from the supernatant using one round of preparative HPLC, and two rounds of analytical HPLC using conditions described in *SI Appendix*, Materials and Methods.

**Plant Material and Bacterial Growth.** *A. thaliana* was grown in walk-in growth rooms maintained at 21 °C/18 °C (day/night) with a 9-h/15-h (day/night) cycle. *N. benthamiana* was grown in a walk-in growth room maintained at





**Fig. 5.** Effect of the phevamines and prephevamine on the spermidine potentiation of the ROS burst and suppression of the calcium burst. (A) Phevamine A suppresses the spermidine potentiation of the flg22-induced ROS burst in *N. benthamiana*, but does not affect the flg22-induced ROS burst directly. Leaf disks were treated with 50 nM flg22, with or without spermidine at 400  $\mu$ M, and with or without phevamine A at 400  $\mu$ M. (B) Phevamine A and prephevamine suppress the spermidine potentiation in *N. benthamiana*, but phevamine B does not. Leaf disks were treated with flg22 at 10 nM, spermidine, phevamines, and prephevamine were used at 300  $\mu$ M. (C) In *Arabidopsis*, phevamine A suppresses the spermidine potentiation, while prephevamine does not. Leaf disks were treated with flg22 at 10 nM, spermidine and phevamines were used at 300  $\mu$ M. (D) Phevamine A does not affect the flg22-induced Ca<sup>2+</sup> burst, or the reduction of this burst by spermidine. Leaf disks were treated with flg22 at 20 nM, spermidine and phevamine A were used at 300  $\mu$ M. Error bars represent SEs. PHVA, phevamine A; PHVB, phevamine B; pPHV, prephevamine; RLU, relative luminescence units; Spd, spermidine. All experiments presented here were repeated at least three times.

26 °C/22 °C with a 12-h/12-h (day/night) cycle and a LGM550 professional LED grow light system (LED Grow Master Global). For maintenance and transformation, *Pseudomonas* strains were grown in King's B media at 28 °C. For infiltration or dipping *in planta*, *Pseudomonas* strains were grown in liquid culture overnight with the appropriate antibiotics, then plated on a Petri dish and incubated overnight before resuspension in 10 mM MgCl<sub>2</sub>.

**Bacterial Growth Assay.** *Pto*, *Pto* $\Delta$ hsv, *Pto*-Cor<sup>-</sup>, and *Pto*-Cor<sup>-</sup> $\Delta$ hsv cells were resuspended in 10 mM MgCl<sub>2</sub> to a final concentration of  $2 \times 10^5$  cfu/mL. Twenty-day-old *A. thaliana* Col-0 seedlings were dipped in bacterial solutions supplemented with Silwet L-77 (Momentum), and growth was assessed at day 0 and 3-d postinfection as described by Törner and Dangl (57). Eight samples containing three seedlings were collected for each treatment.

**Callose Deposition Measurement.** Three- to 5-wk-old *Arabidopsis* plants were infiltrated with *Pseudomonas* strains at an OD<sub>600</sub> of 0.2 in 10 mM MgCl<sub>2</sub> and collected after about 20 h. To visualize callose deposition, leaves were stained with aniline blue (58). The tissue was cleared and dehydrated with 96% ethanol overnight at 37 °C. Cleared leaves were washed with distilled water and then stained in 0.01% aniline blue in 150 mM K<sub>2</sub>HPO<sub>4</sub> (pH 9.5) for

4 h at room temperature. Stained samples were washed and mounted in distilled water and examined by epifluorescence (LEICA M205 FA) with 100 $\times$  magnification. Images were taken at the region below the infiltrated zone of each leaf. Counting of accumulated callose foci was carried out using ImageJ (NIH). For each treatment, 10–20 leaves were processed.

**Phylogenetic Analysis.** The phylogenetic history of *P. syringae* was inferred from Yang et al. (37). Protein sequences of toxin biosynthetic genes listed in Dataset S1 were used as basic local alignment search tool (BLAST) queries to search for homologous protein sequences in the 292 *P. syringae* genomes [available as of January 2017, Pathosystems Resource Integration Center (PATRIC)]. A hit with over 80% protein sequence identity was considered positive. The tree was visualized using interactive tree of life (iTOL) (59).

**ROS Burst Measurement.** Leaf disks from 4-wk-old *A. thaliana* Col-0, or *N. benthamiana* were placed into a 96-well plate with 100  $\mu$ l of water in each well. Twelve leaf disks were used per treatment. After overnight incubation for *Arabidopsis* leaf disks, or 20 h for *N. benthamiana* leaf disks, each sample was treated with 100  $\mu$ l of reaction mix, including 17 mg/mL of luminol (Sigma), 10 mg/mL of horseradish peroxidase (Sigma), distilled water, flg22 at

concentrations ranging from 5 to 100 nM, L-arginine, and polyamines at concentrations typically ranging from 200 to 800  $\mu$ M. Phevamines and pre-phevamine were used at the same concentration as L-arginine (Sigma) or spermidine. Luminescence was measured immediately with 0.5-s integration and 1-min interval over 45 min using a SpectraMax L (Molecular Devices). For each experiment, 8–12 leaf-disks per condition were monitored. The flg22 peptide (QRLSTGSRNSAKDDAAGLQIA) was synthesized by Genscript (41).

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